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INFLUENCE OF BOVINE SEMINAL PLASMA ON EPIDIDYMAL SEMEN FROM THE AFRICAN BUFFALO (*Syncerus caffer*) FROZEN WITH TRILADYL™ OR ANDROMED®

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Abstract

Numerous diseases are carried and can be transmitted from the African buffalo (*Syncerus caffer*) to livestock. Therefore, buffaloes may only be moved with a special transport permit. Disease-free buffaloes are in demand amongst private game farmers. Current disease-free animals derive from a small genetic pool and hence there is a special interest in bringing new genetic material into the disease-free populations. Different breeding programs were developed in the past, which allow producing disease-free offspring from an infected herd.
In this study epididymal sperm from 16 mature African buffalo bulls were frozen with Triladyl™ and AndroMed® (both Minitüb, Germany) with or without the addition of bovine seminal plasma. Post-thaw motility, longevity and acrosomal integrity were compared by means of paired two-tailed t-tests.
For both cryodiluents the post-thaw motility was mostly higher when no seminal plasma was added: no differences could be seen for the acrosomal integrity. Triladyl™ was superior to AndroMed® in regards to total post-thaw motility.
This study indicates that the use of bovine seminal plasma in a concentration of 10% is detrimental rather than beneficial in regards to the post-thaw motility. Triladyl™ rather than AndroMed® should be used to freeze buffalo epididymal sperm, since it is superior in terms of post-thaw motility, even though the former, containing egg yolk, is not a defined medium and therefore lacks quality standards and carries a hygiene risk.

Zusammenfassung

Der afrikanische Büffel (*Syncerus caffer*) stellt als Träger verschiedener Krankheiten ein Infektionsrisiko für den Hausviehbestand dar, weswegen der Transport strengen Reglementierungen unterliegt. Aufgrund dessen gibt es eine besondere Nachfrage unter Wildzüchtern nach krankheitsfreien Büffeln. Da der Großteil solcher Tiere aber von einer relativ kleinen Ursprungspopulation stammt ist der Bedarf gegeben neues genetisches Material einzubringen. Verschiedene Zuchtprogramme wurden bisher etabliert um Büffel krankheitsfrei zu „produzieren".
In dieser Studie wurde epididymales Sperma von 16 ausgewachsenen Büffeln mit Triladyl™ und AndroMed® (beide Minitüb, Deutschland) sowohl mit als auch ohne der Zugabe von seminalem Plasma gefroren. Die Spermaqualität wurde nach dem Auftauen anhand von Beweglichkeit, Unversehrtheit der Akrosomen und Langlebigkeit, mit Hilfe von gepaarten t-tests ermittelt.
Es ergab sich bei der Verwendung beider Gefriermedien jeweils eine höherer Beweglichkeit, wenn kein seminales Plasma zugesetzt war. Die Akrosomen waren bei allen verwendeten Behandlungen in gleichem Ausmaß geschädigt. In Bezug auf die totale Beweglichkeit der Spermatozoa nach dem Auftauen, war Triladyl™ das Medium der Wahl.
Die Ergebnisse dieser Studie weisen darauf hin, dass ein Zusatz von 10% seminalem Plasma die Beweglichkeit von Spermienzellen schädigt. Es zeigt sich weiterhin, dass trotz eines gewissen Hygienerisikos, welches sich aus dem Zusatz von Eigelb ergibt, Triladyl™ derzeit das Mittel der Wahl zur Tiefgefrierung von Büffelsperma darstellt.

Résumé

Le buffle africain (*Syncerus caffer*) peut être porteur de nombreuses maladies et peut également en transmettre au bétail. Aussi les bisons ne doivent-ils être déplacés qu'avec un permis de transport spécial. La demande de bisons indemnes de maladies est conséquente chez les éleveurs de gibier privé. Les animaux indemnes de maladies aujourd'hui disponibles sont issus d'une population restreinte, et il est donc intéressant d'apporter de

la diversité génétique à la population indemne. Divers programmes d'élevage ont été développés dans le passé, permettant d'obtenir une descendance indemne à partir d'une population initialement infectée.
Dans cette étude, le sperme épididymal de 16 mâles bisons sexuellement matures a été congelé avec Triladyl TM et Andromed â (Minitub, Allemagne) avec ou sans addition de plasma séminal bovin. La motilité post-congélation, la longévité et l'intégrité de l'acrosome ont été comparés au moyen de two-tailed t-tests pariés. Avec les deux cryodiluants la motilité post-congélation était notamment supérieure en l'absence de plasma, mais aucune différence n'a été détectée en ce qui conceme l'intégrité . Triladyl TM s'est montré plus efficace qu' Andromed â pour préserver la motilité post-congélation.
Cette étude montre que l'utilisation decplasma séminal bovin à la concentration de 10% est plus nuisible que bénéfique en ce qui concerne lamotilité post-congélation. Triladyl TM devrait être préféré à Andromed â pour congeler le sperme épididymal de bison, puisqu'il est meilleur en ce qui concerne la motilité post-congélation, même s'il contient du jaune d'œuf et n'est pas un milieu défini (pas de standards de qualité), ce qui sous-entend un risque hygienique.

Key words: African buffalo, epididymal spermatozoa, cryopreservation, seminal plasma

Introduction

The African buffalo (*Syncerus caffer*) belongs to Africa's so called "big five". It is therefore a popular animal amongst hunters, game viewers and above all game farmers. Fortunately the African buffalo is not considered an endangered species within the Republic of South Africa and the numbers are very favourable. Many of the buffalo populations in Southern Africa are infested with corridor disease (CD), tuberculosis, brucellosis or foot-and-mouth-disease (FMD). The demand for so-called "disease free" animals in the private sector exceeds the availability at the moment (10).
The African buffalo is believed to be the only long term carrier and reservoir for SAT strains of the FMD-viruses, which are the causative agents of FMD in South Africa (6), (16). Transmission of FMD between buffaloes and cattle is not a common event (2), (6) and u nder normal field conditions only buffaloes in an acute stage of the infection are likely to infect domestic cattle, provided that close contact occurs (11). In a study described by Bastos (4) SAT-3 virus was isolated from the semen of an African buffalo, which did not show any clinical signs of the disease. Virus isolation was however never described from epididymal sperm.
Bovine tuberculosis caused by *Mycobacterium bovis* is an exotic disease in Southern Africa and was first brought into the region by English and Dutch cattle during colonisation in the 19th century (27). The first time the disease was detected in South Africa in African buffaloes was in 1990 in the Krüger National Park (KNP). Cases of Corridor disease, which is a buffalo-associated theileriosis, in cattle herds adjacent to the KNP in the 1960s and again in the early 1980s give indications that close contact between cattle and buffaloes took place. Since outbreaks of tuberculosis in these cattle herds were reported at the same time it is like ly that this was the time when tuberculosis was first brought into the National Park (5). Epidemiological surveys on bovine tuberculosis in buffaloes have been conducted since 1993 in the Hluhluwe/Umfolozi National Park. Prevalences exceeded 70% in the worst affected herds (personal communication Jolles). Although never described in African buffaloes, genital tuberculosis occurs in cattle (25).
African buffaloes are also considered to be a source of re-infection of *Brucella abortus* for domestic stock (17). This disease, which can induce abortion and in male animals orchitis, is transmitted by foetuses, foetal membranes, placental fluids, placenta and semen (25).
Another important aspect, in particular related to transport and translocation of African buffaloes, is that they are the only ruminants that are carriers for Corridor disease, caused by *Theileria parva lawrencia* (24) and transmitted by the ticks *Rhipicephalus appendiculatus* and *R. zambeziensis*.
Buffaloes that live in areas, where the above-mentioned diseases are endemic are subject to restrictions of transport. This is to secure the status of the Republic of South Africa as a partly FMD free country, which ensures export of beef, and to prevent spreading of the other diseases.
The introduction of Rinderpest reduced the former large numbers of African buffaloes in South Africa to small populations in the KNP, the Hluhluwe and Umfolozi Nature Reserves and the Addo Elephant National Park by the turn of the 19th century. As the Addo population was the only one free of FMD and Corridor disease, they provided the foundation for the now existing "disease-free"

buffalo population (10), which lead to a relatively small genetic diversity among these animals. As the animals of the Krüger National Park and the Umfolozi/Hluhluwe reserve complex originate from a large genetic pool and are generally larger than buffaloes found elsewhere in the country (12) there is a special desire to reap the benefits of these genetic pools.

Different attempts such as breeding programs and assisted reproductive technologies have been made to produce disease-free African buffaloes. Breeding programs, artificial insemination (AI) and in-vitro fertilisation (IVF) must all have the aim of minimising the risk of spreading the above-mentioned diseases. Whereas *T. parva lawrencia* is only transmitted by ticks and not present in the semen, SAT viruses as well as *B. abortus* can be present. It is however possible to isolate these infectious agents from the semen and to ensure that only non-infected semen is used. Since it is known that there is a genital form of tuberculosis in cattle it is not out of question that it can occur in African buffaloes as well. Tubercular lesions are not likely to be overseen in the testes and epididymides while harvesting epididymal sperm, and therefore it is unlikely to transmit this disease via AI or IVF. To ensure that TB is not transmitted with the semen it would furthermore be advisable to confirm that the donor animals are negative for TB before the semen is used.

Attempts to freeze epididymal semen from African buffaloes have been made in the past. Cryodiluents used were sperm-Tyrodes's-albumin-lactat-pyrovate (sperm-TALP) (3), (20) and Triladyl™ (Minitüb, Germany) (3), (20) and (13). The total post-thaw motilities achieved in these studies have been between 19% and 40%.

Various researchers have tested in different domestic animal species whether or not the presence of seminal plasma has a beneficial effect on the post-thaw quality of spermatozoa. Maxwell describes an increase in the percentage of live as well as of motile spermatozoa after using a semen extender with a content of 10% seminal plasma in rams, boars and bulls, compared to the same extender without seminal plasma (22). This confirms prior findings of Graham for rams (14), but he also found that the addition of seminal plasma has no effect on the motility of epididymal bull spermatozoa. Berger (7) reports that pre-incubation of porcine epididymal sperm in seminal plasma protects the acrosomal membranes from damage during cold shock.

As most semen diluents contain egg yolk, there are always hygiene risks. Studies performed by Hartmann show that egg yolk, as well as milk, contains steroid hormones and their precursors (15). These hormones, and the lack of quality standards, may be responsible for a decreased sperm fertilising capacity. In a controlled study sperm parameters and fertility with an egg yolk-free semen diluent (AndroMed ® Minitüb, Germany) in cattle were comparable to the results obtained with an egg yolk containing diluent (Triladyl™) (23).

The aim of this study was to test whether AndroMed® or Triladyl™ are superior to freeze epididymal sperm from the African buffalo and if the addition of bovine seminal plasma before freezing improves post-thaw semen parameters, such as motility, longevity and acrosomal integrity.

Materials and methods

Experimental animals

Testes were collected from 16 African buffalo bulls culled during disease eradication programs in June/July 2001 in Hluhluwe/Umfolozi Game Park, which is under the responsibility of KwaZulu Natal Nature Conservation Service. All animals were killed by rifle shot and only bulls, which had 3 or more pairs of permanent lower incisors (about 3 to 3.5 years of age), were used in the trial.

Collection, processing and evaluation of material

Testes and epididymides from each bull were collected through a scrotal incision within one hour of culling. The vas deferens, the tail of the epididymis and part of its the body were dissected free from each testis and put into a plastic bag. These bags were transported at room temperature to a temporary established laboratory about 30 minutes away from the culling site. All epididymides were further processed within 9 hours of culling. The epididymal duct was dissected free and cut at the site in the tail of the epididymis where the tubular diameter becomes distinctly larger distally. A blunted 23 or 25 G needle connected to a 10 ml syringe was then inserted into the vas deferens and the semen flushed in a retrograde direction from the tail of the epididymis.

The first epididymis removed from the bag was flushed with 5 ml of Triladyl™, while the second was flushed with 5 ml of AndroMed®. The sperm concentration was determined with a haemocytometer.
Each dilution was split in two equal samples A and B. These samples were diluted to a final concentration of 100 x 10^6/ml in such a manner that sample A consisted of semen extender and semen, while sample B consisted of 10% bovine seminal plasma, semen extender and semen. Semen from a *Bos taurus* bull had been collected with an artificial vagina and centrifuged at 600 g for 15 minutes. The supernatant seminal plasma was harvested and frozen. Five 0.25 ml French straws were filled from each one of the four solutions, sealed with plugs of different colours, submerged into a water bottle of 500 ml at room temperature, and placed in a fridge at 4°C. After an equilibration period of 4 hours the straws were taken out of the water and dried, avoiding warming up, before being put 4 cm above liquid nitrogen in the vapour phase. After ten minutes they were plunged into the liquid phase and stored in goblets in a liquid nitrogen container.
100 µl of every one of the four solutions were further diluted with 400 µl of the corresponding diluent. A drop of 7 µl was placed on a microscopic slide, covered with a 22x22 mm coverslip and put on a pre-warmed heating stage. Percentage of total and progressive motility was determined by eyeball assessment using phase contrast microscopy and a 200 times magnification.
Evaluation of the post-thaw semen quality
After a storage time of about two months the semen was thawed in a water bath of 37°C for at least 30 seconds. The contents of two straws of the same treatment were emptied into two different pre-heated (37°C) 3 ml Perspex-tubes and stored in a water-bath at 37°C.

Motility and longevity
25 µl of the thawed semen were further diluted with 100 µl of the corresponding diluent to determine the motility. The motility of the semen from both thawed straws was determined. In the rare event of remarkable differences this was attributed to handling, and the higher motility was recorded.
The motility was assessed at one and two hours after thawing to determine the longevity.

Acrosomal integrity
A semen smear was made after thawing, before the solution was further diluted for motility assessments, and stained with Eosin/Nigrosin. The sperm morphology was evaluated using a phase contrast microscope (x1000 magnification). 200 sperm cells of every smear were evaluated. The number of spermatozoa with an intact acrosome was expressed as a percentage.

Statistical analysis
The four different treatments used in this trial (A: Triladyl™, B: Triladyl™ with seminal plasma, C: AndroMed®, D: AndroMed® with seminal plasma) were compared by means of paired two-tailed t-tests in the following ways: Treatment A and B were compared with each other as well as treatment C and D to test for the influence of seminal plasma, when sperm was frozen with Triladyl™ or AndroMed® respectively.
Furthermore treatments A and C and treatments B and D were compared to test for the influence of freezing diluent when sperm was frozen with Triladyl™ or AndroMed® with or without the addition of seminal plasma.

Results

The use of Triladyl™ without the addition of seminal plasma (treatment A) resulted in a higher total motility before freezing and immediately after thawing ($p<0.01$) and in a higher post-thaw progressive motility ($p<0.01$) compared to Triladyl™ with seminal plasma (treatment B). One hour after thawing there was a significant difference for the total motility only ($p<0.05$) and no difference could be seen two hours after thawing (Table 1).
The differences in total and progressive motility between treatment C and D (AndroMed® without and with seminal plasma) were highly significant before freezing ($p<0.01$), showing higher motility when no seminal plasma was added. After thawing the progressive motility of treatment C was

Table 1: comparison of motility when Triladyl™ was used without (treatment A) and with seminal plasma (treatment B) with a two-tailed paired t-test.

treatment		mean ± SD for motility in %			
		fresh	0 h post-thaw	1 h post-thaw	2 h post-thaw
A	total motility	48.1 ± 10.63	56.4 ± 15.77	58.0 ± 14.54	45.7 ± 17.48
B		35.0 ± 16.33	46.1 ± 17.98	44.3 ± 22.92	43.8 ± 18.20
p value		<0.01	<0.0.1	<0.05	-
A	progressive motility	21.6 ± 12.34	21.7 ± 16.17	21.1 ± 16.75	12.2 ± 13.48
B		13.4 ± 10.12	12. 6 ± 13.27	18.3 ± 15.18	7.4 ± 11.90
p value		<0.05	<0.0.1	-	-

Table 2 comparison of motility when AndroMed® was used without (treatment C) and with seminal plasma (treatment D) with a two-tailed paired t-test.

treatment		mean ± SD for motility in %			
		fresh	0 h post-thaw	1 h post-thaw	2 h post-thaw
C	total motility	52.5 ± 12.25	45.1 ± 21.85	39.8 ± 24.53	37.4 ± 19.26
D		19.4 ± 17.22	47.8 ± 20.31	38.9 ± 23.39	34.7 ± 18.84
p value		<0.01	-	-	-
C	progressive motility	30.3 ± 18.57	16.3 ± 11.13	13.2 ± 13.27	6.3 ± 10.23
D		8.8 ± 9.22	17.2 ± 14.27	4.6 ± 6.94	0.6 ± 2.5
p value		<0.01	-	<0.05	<0.1

Table 3 comparison of motility when Triladyl™ (treatment A) and AndroMed® (treatment C) were used without seminal plasma with a two-tailed paired t-test.

treatment		mean ± SD for motility in %			
		fresh	0 h post-thaw	1 h post-thaw	2 h post-thaw
A	total motility	48.1 ± 10.63	56.4 ± 15.77	58.0 ± 14.54	45.7 ± 17.48
C		52.5 ± 12.25	45.1 ± 21.85	39.8 ± 24.53	37.4 ± 19.26
p value		-	<0.05	<0.01	<0.05
A	progressive motility	21.6 ± 12.34	21.7 ± 16.17	21.1 ± 16.75	12.2 ± 13.48
C		30.3 ± 18.57	16.3 ± 11.13	13.2 ± 13.27	6.3 ± 10.23
p value		<0.05	-	-	-

Table 4 comparison of motility when Triladyl™ (treatment B) and AndroMed® (treatment D) were used with addition of seminal plasma with a two-tailed paired t-test.

treatment		mean ± SD for motility in %			
		fresh	0 h post-thaw	1 h post-thaw	2 h post-thaw
B	total motility	35.0 ± 16.33	46.1 ± 17.98	44.3 ± 22.92	43.8 ± 18.20
D		19.4 ± 17.22	47.8 ± 20.31	38.9 ± 23.39	34.7 ± 18.84
p value		<0.01	-	-	<0.01
B	progressive motility	13.4 ± 10.12	12. 6 ± 13.27	18.3 ± 15.18	7.4 ± 11.90
D		8.8 ± 9.22	17.2 ± 14.27	4.6 ± 6.94	0.6 ± 2.5
p value		-	-	<0.01	<0.05

Table 5 Post-thaw means of intact acrosomes using the 4 different treatments and standard deviation (SD); SP=seminal plasma

	Triladyl™	Triladyl™ + SP	AndroMed®	AndroMed®+SP
	treatment A	treatment B	treatment C	treatment D
average of intact acrosomes in %	63.88	62.54	64.25	63.44
SD	4.23	5.07	3.67	8.17

significantly higher after one hour ($p<0.05$) and showed a trend after two hours ($p<0.05$) (Table 2).
Without the addition of seminal plasma the total post-thaw motility was higher for Triladyl™ than for AndroMed® immediately, one and two hours after thawing ($p<0.05$, $p<0.01$ and $p<0.05$ respectively). The progressive motility was also higher at all times, although the difference was not significant (Table 3).
When seminal plasma was added to both cryodiluents there was a highly significant higher ($p<0.01$) total motility and a higher (but not significant) progressive motility in fresh semen, when Triladyl™ was used. After thawing the progressive motility for Triladyl™ was significantly higher after one ($p<0.01$) and two hours ($p<0.05$) and the total motility was significantly higher after two hours ($p<0.01$) (Table 4).
Diluent and seminal plasma had no effect on the post-thaw acrosomal integrity (Table 5).

Discussion

The motility of spermatozoa from the two epididymides of *Bos taurus* bulls does not differ (19), therefore the use of a different cryodiluent for each epididymis was regarded to be adequate to compare the effect of these diluents on sperm parameters.
The results of the present study show that the post-thaw motility of epididymal African buffalo spermatozoa cannot be increased by adding seminal plasma prior to freezing. This is at least true for both of the cryodiluents used in this experiment, namely Triladyl™ and AndroMed®. Concurrent results on the influence of seminal plasma are reported for some domestic species. Magistrini describes a lower post-thaw motility for stallion ejaculated sperm cells than for epididymal spermatozoa (21). Seminal plasma is detrimental for the motility of fresh epididymal spermatozoa in boars (7). Graham (14) reported that the immediate post-thaw motility did not differ in cattle whether seminal plasma was added prior to freezing or not, when a TRIS-sodium-glucose-egg-yolk-glycerol extender was used. These results are in correspondence with the semen frozen with

AndroMed®, but not with the semen frozen with Triladyl™, where the immediate post-thaw motility was better if no seminal plasma was added. The longevity, which is not reported in Graham's trial, was better for both, AndroMed® and Triladyl™ if no seminal plasma was added. For AndroMed® the difference increased over time, whereas it was no longer significant for Triladyl™ after two hours of incubation. This indicates that seminal plasma negatively influenced the rate of decrease if semen was frozen with AndroMed®.

In contrast to the present study Maxwell reported an increase in the percentage of live as well as progressive motile spermatozoa after using an extender with a content of 10% seminal plasma in fresh bull, ram and boar semen compared to the same extender without seminal plasma (22). The differences in his results compared to those of Graham and the ones we recorded for the African buffalo, might be explained by the fact that Maxwell did not use epididymal, but ejaculated semen.

It was expected that the addition of seminal plasma to fresh epididymal sperm would have a positive effect on the motility prior to freezing. It had, however, a negative effect before and after with both diluents. This stands in contrast to the report of Braun (9), where autologous seminal plasma in stallions had a positive effect on the motility prior to freezing, but a negative effect after freezing and thawing on epididymal semen. A possible explanation for these opposite findings cannot only be found in the different species used. Braun used autologous seminal plasma; this is usually not possible in the African buffalo. Further he found the positive effect only if the seminal plasma was added at a final concentration of 25%, but not if it was only 5%. The 10% seminal plasma in the present study might have been too low to detect a possible positive effect.

Ahmad (1) reported a significantly higher survivability of fresh ejaculated Nili-Ravi buffalo bull spermatozoa incubated at 37°C if the semen was deprived of seminal plasma. Berger found that the acrosomal membranes of epididymal porcine spermatozoa were more resistant to damage of cold shock than those of ejaculated sperm, measured by hyaluronidase release The resistance of epididymal spermatozoa to cold shock could be even more increased by the addition of seminal plasma (7). These findings concur with those of Johnson, who described in equines a higher membrane permeability of eosin for ejaculated than for epididymal spermatozoa (18). In contradiction to the findings of Berger, Bialy (8) reports that spermatozoa became susceptible to cold shock only after being in contact with substances that are present in the secretion of the ampullae. The results reported by Wales (26) that ejaculated sperm is more susceptible to cold shock than epididymal sperm are in accordance to the findings of Bialy. According to Bialy the reason for the increase of the susceptibility is the contact of the sperm with secretion from the ampullae. According to Wales's opinion it may be due to dehydration of the lipid capsule during maturation. He further states that the resistance to cold shock is mainly a property of the cell and is little affected by the secretions of the accessory sex glands.

The study of Berger (7) is the only report where epididymal spermatozoa with or without the addition of seminal plasma and not epididymal and ejaculated spermatozoa are compared for acrosomal damage; the experimental procedures were therefore similar to the ones in our trial. The results of our experiments differ most likely due to the different species used. In the present study there was very little difference in the acrosomal integrity (62.54% to 64.25%) whether Triladyl™ or AndroMed® with or without seminal plasma were used.

Another essential part of this study was to test the possible use of AndroMed®, an egg yolk free cryodiluent, for freezing epididymal semen from the African buffalo. Since egg yolk varies in its constitution there is always a lack of quality standard as well as possible hygiene risks. These factors together with steroid hormones and their precursors, which are also present in egg yolk (15), were mentioned by Müller-Schlösser (23) as possible reasons for a decrease of sperm parameters if diluents containing egg yolk are used. When post-thaw quality of bovine semen frozen with AndroMed® was compared to semen frozen with a standard TRIS-egg yolk-diluent no differences in motion parameters and the inducibility of the acrosome reaction were found. In the present study the post-thaw motility was always better if sperm was frozen with Triladyl™ than if it was frozen with AndroMed®, although the difference was not always significant.

It can be concluded that the addition of bovine seminal plasma in a final concentration of 10% to either Triladyl™ or AndroMed® does not improve the post-thaw motility, the longevity or the acrosomal integrity of epididymal sperm from the African buffalo and that Triladyl™ is superior to AndroMed® to freeze such sperm.

References

1. Ahmad, M., Khan, A., Shah, Z. A., and Ahmad, K. M. Effects of removal of seminal plasma on the survival rate of buffalo bull spermatozoa. Animal Reproduction Science 1996;41:193-9.
2. Anderson, E. C. Potential for the transmission of foot-and-mouth disease virus from African buffalo (*Syncerus caffer*) to cattle. Research in Veterinary Science 1986;40:278-80.
3. Bartels, P., Lambrechts, H., Kidson, A., and Friedmann, Y. The potential of breeding disease-free African buffalo using assisted reproductive technology. Proceedings of a Symposium on the African Buffalo as a Game Ranch Animal 1996;75-8.
4. Bastos, A. D. S., Bertschinger, H. J., Cordel, C., van Vuuren, C. de W. J., Keet, D., Bengis, R. G., Grobler, D. G., and Thomson, G. R. Possibility of sexual transmission of foot-and-mouth disease from African buffalo to cattle. Veterinary Record 1999;145:77-9.
5. Bengis, R. G., Kriek, N. P. J., Keet, D. F., Raath, J. P., Vos, V. de, Huchzermeyer, H. F. A. K., and De, Vos, V. An outbreak of bovine tuberculosis in a free-living African buffalo (*Syncerus caffer*-Sparrman) population in the Kruger National Park: a preliminary report. Onderstepoort Journal of Veterinary Research 1996;63:15-8.
6. Bengis, R. G., Thomson, G. R., and De Vos, V. Foot and mouth disease and the African buffalo: a review. Journal of the South African Veterinary Association 1987;58:160-2.
7. Berger, T. and Clegg, E. D. Effect of male accessory gland secretion on sensitivity of porcine sperm acrosomes to cold shock, initiation motility and loss of cytoplasmatic droplets. Journal of Animal Science 1985;60:1295-1302.
8. Bialy, G. and Smith, V. R. Cold shock of epididymal spermatozoa. Journal of Dairy Science 1959;42:2002-
9. Braun, J., Sakai, M., Hochi, S., and Oguri, N. Preservation of ejaculated and epididymal stallion spermatozoa by cooling and freezing. Theriogenology 1994;41:809-18.
10. De Vos, V. The status and distribution of the buffalo (*Syncerus caffer*) in South Africa. Journal of the South African Veterinary Association 1987;58:157-
11. Gainaru, M. D., Thomson, G. R., Bengis, R. G., Esterhuysen, J. J., Bruce, W., and Pini, A. Foot-and-mouth disease and the African buffalo (*Syncerus caffer*). II. Virus excretion and transmission during acute infection. Onderstepoort Journal of Veterinary Research 1986;53:75-85.
12. Gerber, D. Breeding "disease free" African buffalos (*Syncerus caffer*). Proceedings of the Symposium on Wildlife Utilization in Southern Africa . 2000. Pretoria, Faculty of Veterinary Science - University of Pretoria. 2000. Ref Type: Conference Proceeding
13. Gerber, D., Irons, P. C., Arlotto, A., and Cooper, D. Quality and freezability of epididymal semen from African buffalo (*Syncerus caffer*) under field conditions. Theriogenology 2001;55:384-
14. Graham, J. K. Effect of seminal plasma on the motility of epididymal and ejaculated spermatozoa of the ram and bull during the cryopreservation process. Theriogenology 1994;41:1151-62.
15. Hartmann, S., Lacorn, M., and Steinhardt, H. Natural occurrence of steroid hormones in food. Food Chemistry 1998;62:7-20.
16. Hedger, R. S. and Condy, J. B. Transmission of foot-and-mouth disease from African buffalo virus carriers to bovines. Veterinary Record 1985;117:205-
17. Herr, S. and Marshall, C. Brucellosis in free-living African buffalo (*Syncerus caffer*): a serological survey. Onderstepoort Journal of Veterinary Research 1981;48:133-4.
18. Johnson, L., Amann, R. P., and Picket, B. W. Maturation of equine epididymal spermatozoa. American Journal of Veterinary Research 1980;41:1190-96.
19. König, G. J. Überprüfung der Qualitätsveränderungen von Nebenhodenspermien post mortem bei Stieren. 1998. UniversityofVeterinaryMedicineVienna. Ref Type: Thesis/Dissertation
20. Lambrechts, H., van Niekerk, F. E., Coetzer, W. A., Cloete, S. W. P., and van der Horst G. The effect of cryopreservation on the survivability, viability and motility of epididymal African buffalo (*Syncerus caffer*) spermatozoa. Theriogenology 1999;52:1241-49.
21. Magistrini, M., Tinel, C., Noue, P., and Palmer, E. Correlations between characteristics of frozen spermatozoa from ejaculates or perfusates from epididymidis caudae and proximal deferent ducts in a group of stallions. 11th International Congress on Animal Reproduction and Artificial Insemination, University College Dublin, Ireland, June 26 30 1988 1988;273:
22. Maxwell, W. M. C., Welch, G. R., and Johnson, L. A. Viability and membrane integrity of spermatozoa after dilution and flow cytometric sorting in the presence or absence of seminal plasma. Reproduction, Fertility and Development 1996;8:1165-78.
23. Müller-Schlösser, F., Aires, V., Hinsch, E., and Hinsch, K.-D. Evaluation of the quality of a new generation of egg yolk-free semen diluters for cryopreservation of bovine semen. Reproduction of Domestic Animals 2001;in print:
24. Potgieter, F. T., Stoltsz, W. H., Blouin, E. F., and Roos, J. A. Corridor disease in South Africa: a review of the current status. Journal of the South African Veterinary Association 1988;59:155-60.
25. Rosenberger, G. Infektionskrankheiten. In: Krankheiten des Rindes. Berlin,Hamburg: Paul Parey Verlag, 856-873.
26. Wales, R. G. and White, I. G. The susceptibility of spermatozoa to temperature shock. Journal of Endocrinology 2001;19:211-20.
27. Webb, G. B. Tuberculosis. New York: Clio Medica, quoted by Tanner, M. Investigation of the viability of *M.bovis* under different environmental conditions in the Kruger National Park. Onderstepoort Journal of Veterinary Research 1999;66:185-90.

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BOSS OR NOT? – ENDOCRINOLOGIC EVALUATION OF REINTRODUCED PRZEWALSKI HORSE STALLIONS (*Equus caballus przewalskii*) IN MONGOLIA

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Abstract

The aim of this study was to find out whether it was possible to monitor steroid hormone levels in free roaming Przewalski stallions *(Equus caballus przewalskii)* using faecal sampling as a non-invasive method. Throughout a year faecal samples from four harem stallions and eight bachelor stallions were collected. Faecal extracts were analysed for immunoreactive androgen and oestrogen metabolites. Harem stallions secreted significantly higher levels of estrogens, testosterone and epiandrosterone than bachelor stallions did. For estrogens and epiandrosterone a seasonal pattern with higher levels in spring and summer when compared to autumn and winter was determined. The monitoring of faecal steroid hormone levels in Przewalski stallions is possible using estrogens, 17ß-OH-Androstane and 17-oxo-Androstane assays. Using faecal samples instead of plasma the hormonal status of free-ranging Przewalski stallions can be evaluated in a non-invasive way. The results pertaining to the seasonality of hormone levels and the various faecal hormone levels in bachelor and harem stallions confirm previous findings of plasma testosterone and oestrogen levels in domestic and feral horses.

Zusammenfassung

Ziel der Studie war es, herauszufinden, ob Steroidhormonwerte bei Przewalskihengsten in freier Wildbahn mittels Kotproben nicht-invasiv überwacht werden können. Im Verlaufe eines Jahres wurden Kotproben von vier Leithengsten und acht Hengsten einer Junggesellengruppe gesammelt. Die Kotprobenextrakte wurden auf ihren Gehalt an immunoreaktiven Androgen- und Östrogenmetaboliten hin untersucht. Bei Leithengsten wurden signifikant höhere Gehalte an Östrogenen, Testosteron und Epiandrosteron festgestellt als bei Junggesellenhengsten. Die Kurven für Östrogene und Epiandrosteron zeigten saisonale Schwankungen mit höheren Werten in Frühjahr und Sommer verglichen mit denen in Herbst und Winter.
Die Kontrolle der Steroidhormongehalte im Kot von Przewalskihengsten erweist sich unter Verwendung von Assays für Östrogene, 17ß-OH-Androstane und 17-oxo-Androstane als durchführbar. Durch die Untersuchung von Kotproben anstelle von Plasmaproben wird eine nicht-invasive Erfassung des hormonellen Status bei freilebenden Pferden möglich. Die Ergebnisse bestätigen frühere Studien, die anhand von Plasmauntersuchungen an Hauspferden und verwilderten Hauspferden durchgeführt wurden.

Résumé

Le but de cette étude était de savoir si il était possible de contrôler les niveaux d'hormones stéroïdes chez des étalons chevaux de Przewalski *(Equus caballus przewalski)* en liberté en utilisant les fécès comme méthode non

invasive de prélèvement. Sur une durée de un an, des fécès de quatre étalons en harem et huit célibataires ont été récoltées. La présence d'androgènes immunoréactifs et de métabolite œstrogéniques a été recherchée à partir des extraits fécaux . Les étalons en harem sécrétaient significativement plus d'œstrogènes, de testostérone et d'épiandrostérone que les célibataires. Pour les œstrogènes et l'épiandrostérone, un rythme saisonnier avec des nivaux plus élevés au printemps et en été qu'a l'automne et en hiver a été observé. Le suivi des niveaux d'hormones stéroïdiennes chez les étalon est possible en testant les œstrogènes, le 17b-OH-androstane et le 17-oxo-androstane. En utilisant des fécès au lieu de plasma, le statut hormonal des chevaux de Przewalski mâles peut être évalué de façon non invasive. Les résultats indiquant une saisonnalité des niveaux hormonaux ainsi que la différence des niveaux hormonaux entre les célibataires et les étalons en harem confirment les découvertes précédentes sur les niveaux plasmatiques de testostérone et d'œstrogènes chez les chevaux domestiques et en liberté.

Key words: Przewalski horse, hormones, testosterone, epiandrosterone, oestrogen, faecal analysis.

Extended Abstract

The Przewalski horse became extinct in the wild in the 1960´s. The last recorded sightings took place in the Dzungarian Gobi in the southwest of Mongolia (14). Fortunately, the species survived in zoos. Today the population consists of approximately 2000 Przewalski horses *(Equus caballus przewalskii)* or „Takhis“ in Mongolian, all descendants from only 13 founder animals. In 1992 a reintroduction project was established at the Takhin Tal site (45.53.80 N, 93.65.22 E) on the edge of the 12,500km^2 Gobi B, a strictly protected area and International Biosphere Reserve. In 1999 the International Takhi Group(ITG) was founded in Europe and Mongolia in order to continue the project in accordance with the IUCN reintroduction guidelines.

Since 1992 a total of 59 zoo-born Takhis have been transported to Takhin Tal. The first harem group was released in 1997, the first wild born foals were successfully raised in 1999. In the same year the first bachelor group was established. Thus, in the year 2000 three harem groups and a group of six bachelor stallions ranged throughout the reintroduction site. Another harem group and a group of four newly arrived mares were kept within adaptation enclosures. In the summer of 2000 two young stallions were forced out of a harem group (Pas) and joined the bachelors. The idea of the bachelor group being a refuge for loner stallions seemed to work out.

The aim of this study was to find out whether it was possible to monitor steroid hormone levels in free roaming Przewalski stallions using faecal sampling as a non-invasive method. We wanted to determine if a seasonal pattern in the secretion of the various sexual hormones was present. Furthermore, we wanted to evaluate if in free-ranging Przewalski horses there are a measurable difference between the hormonal statuses of bachelor and harem stallions.

Androgens and estrogens were determined in the faecal samples. The major androgen is testosterone. Mainly the Leydig cells in the testis produce it. Androgens are needed for the development and maintenance of sexual organs and the appearance of secondary sexual characteristics including anabolic effects on the musculoskeletal system. Andrdogens are responsible for the stimulation of the spermatogenesis (2). About 28% of the excreted testosterone is found in the faeces while 72±7% is excreted via urine (15). 17ß-OH-Androstane and 17-oxo-Androstane have been shown to be the principal faecal testosterone metabolites excreted (10). Plasma testosterone concentrations have been measured in a variety of studies in domestic and feral horses. Several authors reported a seasonal pattern for plasma testosterone levels in stallions with high concentrations in spring and summer followed by lower concentrations in winter (1, 4,3, 8, 19). In ponies and in feral horses significantly higher testosterone levels were observed in dominant stallions when compared to bachelor stallions (7, 8). Other steroid hormones that are secreted in large amounts by the testis and some other tissues are estrogens (2, 3, 13). Estrogens occur not only in blood plasma but also in seminal plasma. The importance and possible effects of male estrogens on the female genital tract are not yet understood. Stallions reach higher oestrogen levels in the urine than mares (20). Via faeces, estrogens are excreted mainly as oestradiol-17α or-17ß and as estrone. Only 2% of the estrone is secreted via faeces (15). Similar to testosterone a seasonal pattern has been revealed for oestrogen levels in the plasma of stallions (5, 12). Faecal oestrogen analysis has been used to diagnose equine cryptorchidism in domestic stallions (4, 11).

In this study we examined a total of 128 faecal samples from twelve stallions (four harem stallions and eight bachelor stallions) at the reintroduction site between November 1999 and October 2000. The faeces were stored in ethanol until their extraction for the enzyme-immunoassay (EIA). The method used was the methanol extraction method of Schwarzenberger et al (2000). The faecal extracts were analysed for immunoreactive androgen and oestrogen metabolites. Assays included 17ß-OH-Androstane (trivial name: testosterone), 17-oxo-Androstane (trivial name: epiandrosterone), and total estrogens (9). Due to the different metabolites the results are designated as „androstanes" and „estrogens", respectively. Demonstrating parallelism between standard curves and serial dilutions of faecal extracts validated the assays.

Harem stallions secreted significantly higher levels of estrogens, testosterone ($p<0,001$) and epiandrosterone ($p=0,002$) than bachelor stallions did. For estrogens ($p=0,001$) and epiandrosterone ($p=0,006$). A seasonal pattern with higher levels in spring and summer when compared to autumn and winter was determined. The same seemed to be true for testosterone but could not be proved statistically. In harem stallions, both estrogens and testosterone demonstrated additional peaks in November and October, respectively.

Statistically, the mean levels of faecal estrogens, testosterone and also epiandrosterone are correlated not only with status but also with age (17-ß –OH-androstane $r_s = 0.74$ $p<0.001$; 17 – oxo – androstane $r_s = 0.30$ $p<0.001$; Estrogens $r_s = 0.44$ $p<0.001$). In our study, the excretion of all three sex hormones increased with age from three to eight year-old stallions. The 11 year-old harem stallion „Pas" had significantly lower levels of testosterone than the other harem stallions (t-test $p<0.001$). The mean concentration of this metabolite in "Pas" was similar to those of bachelor stallions. In the 6year-old harem stallion „Uentsch" the mean concentration of faecal epiandrosterone did not differ from that of bachelor stallions (t-test $p>0.05$). In contrast to this, „Uentsch" demonstrated significantly higher concentrations of faecal estrogens when compared to the bachelor stallions (t-test $p<0.01$).
The monitoring of faecal steroid hormone levels in Przewalski stallions is possible using estrogens, 17ß-OH-Androstane and 17-oxo-Androstane assays. Using faecal samples instead of plasma, it is possible to evaluate the hormonal status of free-ranging Przewalski stallions in a non-invasive way. The results pertaining to the seasonality of hormone levels confirm previous findings of plasma testosterone and oestrogen levels in domestic (1, 3, 4, 6, 8, 12) and feral horses (19). The various faecal hormone levels in bachelor and harem stallions agree with a former study on plasma testosterone levels in pony stallions (8). Harem stallions excrete higher amounts of sex hormones than bachelors do. In the studied animals the concentrations of hormones are correlated with age and status. Since in our investigation the harem stallions were significantly older than bachelors, it is difficult to say which factor is the most important. A study of plasma testosterone levels in captured feral stallions supports the idea of the status being a more important factor than age (7). In that study, an eight-year old bachelor stallion had significantly lower levels than two harem stallions. Those findings agree with another study on pony stallions (8). There, bachelor stallions that became harem stallions showed a sharp rise in plasma testosterone. Then, after displacement from harem status, the stallions plasma testosterone level decreased again. The same pattern could be observed in the case of our bachelor stallion „Sondor". His testosterone level rose sharply and was the highest of all bachelors as he started to herd a group of domesticated geldings. One month later, back in the bachelor group, his levels had decreased remarkably. Considering the stallions aged three to eight years in our study, the hormone levels increased with the animal's age. However, the oldest stallion „Pas", who is a successful harem stallion, revealed bachelor-like concentrations of androgens. This suggests that older stallions might be able to compensate for lower production and secretion of hormones with experience. Compared to the other harem stallions, the six-year old „Uentsch" excreted remarkably low levels of all three investigated hormones. For estrogens and testosterone, the difference was not significant. Interestingly, „Uentsch" was the only harem stallion that did not reproduce successfully. Whether this was due to his hormonal status could not be answered in this study. The rise in testosterone levels in winter has also been observed by other authors (1) but has been considered as a result of inadequate sampling frequency. Since it takes about two month to produce fertile sperms the testosterone rise might be the initiation of spermatogenesis. But this does not explain the following decrease. Another possible explanation would be apoptosis of spermatocytes in the testis. Testosterone is known to

be a cell survival factor in the testis (21). The decrease of testosterone production in the non-breeding season could possibly cause apoptosis and thereby a great release of testosterone from the dying cells.

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References

1 Berndtson, W.E., Pickett, B.W.& Nett; T.M. Reproductive physiology of the stallion. IV.Seasonal changes in the testosterone concentration of peripheral plasma. J.Reprod.Fert. 1974; 39: 115-8.
2 Brown, T.R. Steroid Hormones, Overview. In: Knobil, E. and Neill, J.D. (Eds) Encyclopedia of reproduction. Volume 4. Academic Press 1999; 634-44.
3 Döcke, F. Keimdrüsen. In: Döcke, F. (Ed): Veterinärmedizinische Endokrinologie. 3.Aufl. Gustav Fischer Verlag, Jena-Stuttgart, 1994; 399-507.
4 Ganjam, V.K. and Kenney, R.M. Androgens and oestrogens in normal and cryptorchid stallions. J.Reprod.Fert.,Suppl. 1995; 23: 67-73.
5 Goodman, R.L. Seasonal Reproduction, Mammals. In:Knobil, E. and Neill, J.D. (Eds) Encyclopedia of reproduction. Volume 4. Academic Press 1999; 341-51.
6 Hoffmann, B. and Landeck, B. Testicular endocrine function, seasonality and semen quality of the stallion. Animal Reproduction Science 1999; 57: 89-98.
7 Kirkpatrick, J.F., Vail, R., Devous, S., Schwend, S., Baker, C.B. and Wiesner, L. Diurnal Variation of Plasma Testosterone in Wild Stallions. Biol. Reprod. 1976; 15: 98-101.
8 McDonnell, S.M., Murray, S.C. Bachelor and Harem Stallion Behavior and Endocrinology. Biol. Reprod. Mono. 1995; 1: 577-90 .
9 Palme, R. , Möstl, E. Biotin-Streptavidin Enzyme Immunoassay for the Determination of Oestrogens and Androgens in Boar Feaces. Proc. of the 5th Symp. on the Analysis of Steroids, Szombathely, Hungary, 1993; 111-7.
10 Palme, R., Fischer, P., Schildorfer, H., Ismail, M.N. Excretion of infused 14C-steroid hormones via faeces and urine in domestic livestock. Anim. Reprod. Sci. 1996; 43: 43-63.
11 Palme, R., Holzmann, A. and Mitterer, Th:. Measuring faecal oestrogens for the diagnosis cryptorchidism in horses . Theriogenology 1994; 42: 1381-7.
12 Raeside, J.I. Seasonal changes in the concentrations of estrogens and testosterone in the plasma of the stallion. Anim.Reprod. Sci. 1977 /78; 1: 205-12.
13 Raeside, J.I., Christie, H.L. Estrogen concentrations in semen of the stallion. Animal reproduction Science. 1997; 48: 293-300.
14 Ryder, O.A. Genetic analysis of Przewalskis horse in captivity. In: Sokolov, V.E. and Orlov, V.N. (Eds) The Przewalski horse and it`s restoration in nature in Mongolia. Proc. of a FAO/UNDP Meeting, Moscow, CMP GKNT, Moscow; 1988; 50-103 .
15 Schildorfer, H. Ausscheidung von 14C-Steroiden über Kot und Harn bei Ponys; Inaugural-Dissertation, Veterinärmedizinische Universität Wien. 1994.
16 Schwarzenberger, F., Möstl, E., Palme, R., Bamberg, E. Faecal steroid analysis for non-invasive monitoring of reproductive status in farm, wild and zoo animals. Anim. Reprod. Sci.1996; 42: 515-26.
17 Schwarzenberger, F., Palme, R., Bamberg, E., Möstl, E. A review of faecal progesterone metabolite analysis for non-invasive monitoring of reproductive function in mammals. Int. J. Mammal. Biol. 1997; 62 (Suppl.II): 214-21.
18 Schwarzenberger, F., Rietschel, W., Vahala, J., Holeckova, D., Thomas, P., Maltzan, J., Baumgartner, K. and Schaftenaar, W. Fecal Progesterone, Estrogen, and Androgen Metabolites for Noninvasive Monitoring of Reproductive Function in the Female Indian Rhinoceros, *Rhinoceros unicornis.* General and Comparative Endocrinology . 2000; 119, 300-7.
19 Turner, J.W. and Kirkpatrick, J.F. Androgens, behaviour and fertility control in feral stallions. J.Reprod. Fert., Suppl. 1982; 32:79-87.
20 Velle, W. Urinary oestrogens in the male. J. Reprod. Fert. 1966; 12: 65-73.
21 Zirkin, B.R. Spermatogenesis, Hormonal Control of. In: : Knobil, E. and Neill, J.D. (Eds) Encyclopedia of reproduction. Volume 4. Academic Press 1999; 556-63.

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PHARMACOLOGICAL METHODS OF ENHANCING PENILE ERECTION FOR EX-COPULA SEMEN COLLECTION IN STANDING WHITE RHINOCEROS (*Ceratotherium simum simum*)

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Abstract

In a double-blind trial, the influence of alpha-adrenergic agents on manual semen collection in a standing white rhinoceros (*Ceratotherium simum simum*) was evaluated. When comparing the medicated to the non-medicated trials, significant differences in onset, strength and duration of penile erection were noted. Pulsatile contractions of the penile muscles occurred more frequently. The seminal fluid collection was not significantly influenced by drug administration.

Zusammenfassung

Im Rahmen einer Doppelblind-Studie wurde der Einfluss von Medikamenten mit alpha-adrenerger Wirkung auf die manuelle Desemination beim stehenden Breitmaulnashorn (*Ceratotherium simum simum*) untersucht. Beim Vergleich der Versuchseinheiten mit pharmakologischer Unterstützung gegenüber den Placebogaben traten signifikante Unterschiede im Hinblick auf Eintritt, Stärke sowie Dauer der Erektion auf. Pulsatile Bewegungen des Penis nahmen an Häufigkeit zu. Die Gewinnung seminaler Flüssigkeiten wurde jedoch nicht signifikant durch die Medikation beeinflusst.

Résumé

Une étude en double aveugle a permis d'évaluer l'influence des agents alpha adrénergiques sur la récolte manuelle de sperme chez un rhinocéros blanc (*Ceratotherium simum simum)* en station debout. Lorsque l'on compare les essais avec et sans médication, des différences significatives pour le déclenchement, l'intensité et la durée de l'érection ont été notées. Des contractions pulsatiles des muscles péniens étaient plus fréquentes. La collecte de sperme n'était pas significativement influencée par l'administration de médicaments.

Key words: White rhinoceros, *Ceratotherium simum simum*, alpha-adrenergic agonist, detomidine-hydrochloride, butorphanol, erection

Extended Abstract

This study is part of ongoing work toward developing a pharmacological method for ex-copula semen collection in standing white rhinoceros (*Ceratotherium simum simum*). As the common technique to obtain semen samples from rhinoceros is electroejaculation a less invasive procedure for repeated deseminations is required. According to equine literature, alpha-adrenergic agents

can successfully induce ejaculations in domestic stallions (2,3,5). This is sometimes necessary to provide safe semen collection in disabled or injured horses that are unable to mount.
In view of the encouraging results from a previous work (4) we used a combination of Detomidine-Hydrochloride (*Domosedan*®, Pfizer Corporation Austria Ges.m.b.H., A-1071 Wien) and Butorphanol (Butomidor®, Richter Pharma AG, A-5600 Wels) applied IM. While Detomidine-HCl as an alpha-adrenergic agonist predominantly promotes central alpha$_2$-events, the partial opioid agonist Butorphanol enhances the alpha-adrenergic effect (1). Both agents are commonly used in rhinoceros anaesthesia as sedatives and analgesics and are proven to be save in this species (8).

A double-blind trial was conducted on a thirty-year-old white rhinoceros at Salzburg Zoo. The male was conditioned to enter an indoor restraint chute and to tolerate manual stimulation of penis and prepuce (7). The influence of alpha-adrenergic agonists compared to non-medicated trials was evaluated by administering various dosage combinations of Detomidine-HCl/Butorphanol (mg Detomidine-HCl/mg Butorphanol: 8/4, 8/8, 10/4, 10/8, 14/4) and a placebo (sterile water) in a random fashion. The first author and the keeper were blind to the treatment. Prior to IM injection of the standardised volume of 5 ml into the neck muscles, dermal anaesthesia was achieved by applying a eutectic mixture of local anaesthetics (*Emla*®, 5%-Creme, Astra Ges.m.b.H, A-4020 Linz) (6). Manual stimulation consisted of genital massage in cases where no penile prolapse occurred within 15 minutes after injection in order to induce the onset of penile erection. Manually compression of the glans and the base of the penis performed the stimulation of the erect penis. Seminal fluids were collected into pre-warmed graduated containers.
A variety of thirteen different behavioural patterns such as eagerness to enter the chute, fear, nervousness, aggressive and avoidance behaviour during the manipulation, erection strength etc. were assessed independently by the first author and the keeper using questionnaires resembling those described by Wielebnowski (9). Immediately after each session, a coding form with calibrated horizontal lines was rated. Each line was 100 mm long and presented a continuos scale for a particular behavioural item - with minimum and maximum at either end. The score for each item was rated by a vertical crossing. Subsequently, the distance from the minimum side of the line to the position of the cross was measured in millimetres. The distance then represented the numerical score, resulting in scores from 0 to 100.

In addition to the evaluation of subjective behavioural patterns, six objective parameters (time till onset of erection, duration of erection, number of penile contractions, whether seminal fluids could be collected etc. were assessed.

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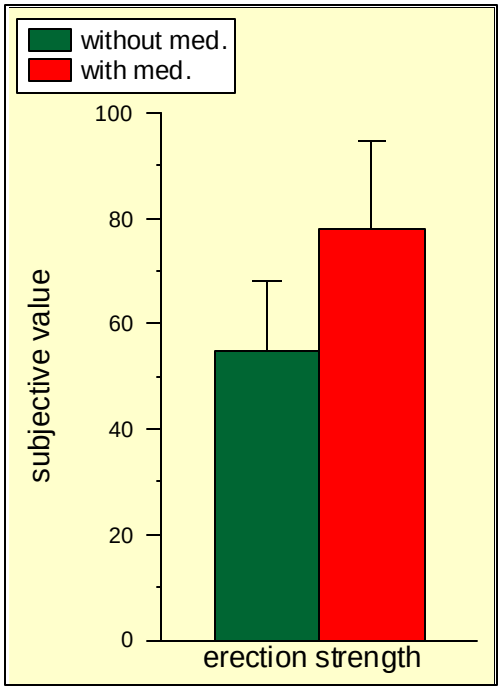

Fig. 1: Pharmacological influence on erection strength. A superior degree of erection was rated in the medicated trials (mean ± SEM). The numerical subjective value score ranges from 0 indicating the minimum to 100 indicating the maximum degree possible.

Comparing the results of the medicated trials to those using a placebo by T-test, the male was scored significantly less aggressive ($p<0,05$), less anxious ($p<0,01$), less nervous ($p<0,01$) and quieter standing ($p<0,01$) in the chute during the manipulation when medication was applied. In addition to those findings that are mainly due to the sedative effect of the applied agents, a superior degree of erection was achieved using the alpha-adrenergic agonists ($p<0,05$) (Figure 1).

Inter-rater reliability was assessed using the T-test and was high for most questionnaire items Seminal fluids were obtained in 25% of the medicated trials compared to 21% of the non-medicated sessions (total number of trials: n=24). With the exception of one ejaculate consisting of three fractions with a total volume of 23 ml and acceptable quality, only small amounts of seminal droplets with poor sperm concentration (<10000/mm^3) could be collected. In most cases, motility and vitality were high (60-85%). For further analysis details on the collected ejaculate with stress on morphologic and morphometric characteristics see Silinski et al. these proceedings. In contrast to the disappointing results concerning our initial aim to allow repeated and predictable manual semen collections, the medication had a significant influence on onset of penile erection ($p<0,05$; T-test) and duration ($p<0,05$; T-test) (Figures 2 and 3).

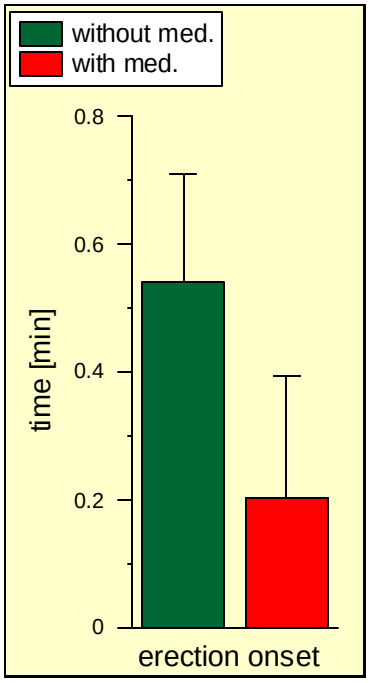

Fig. 2: Onset of penile prolapse and subsequent erection after beginning with manual stimulation in non medicated and medicated trials (mean ± SEM).

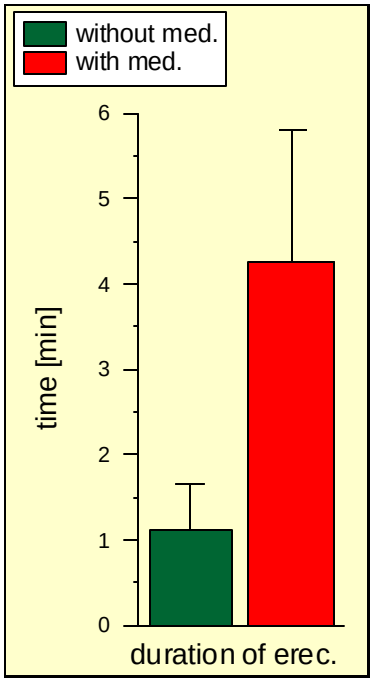

Fig. 3: Duration of penile erection induced by non medicated trials and medicated trials (mean ± SEM).

The induction and number of penile muscle contractions showed a positive trend when alpha-adrenergic agents were administered. Comparing the influence of the various applied doses, no significant difference was noted on the assessed objective parameters.

Though one ejaculate of acceptable quality and quantity was obtained in a medicated trial, a routine collection of semen samples using Detomidine-HCl combined with Butorphanol was not achieved in the evaluated individual.

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References

1. Frey HH, Schulz R and Werner E. Analgetika. In: Frey HH and Löscher W (Eds) Lehrbuch der Pharmakologie und Toxikologie für die Veterinärmedizin. Ferdinand Enke Verlag. Stuttgart 1996; 184-202.
2. McDonnell SM and Love CC. Xylazine-induced ex-copula ejaculation in stallions. Theriogenology 1991; 36: 73-6.
3. McDonnell SM and Odian MJ. Imipramine and xylazine-induced ex copula ejaculation in stallions. Theriogenology 1994; 41: 1005-10.
4. Silinski S, Walzer C, Schwarzenberger F and Stolla R. Influence of alpha-2-agonists on manual semen collection in a standing white rhinoceros (*Ceratotherium simum simum*). Proc. Intern. Elephant and Rhino Res. Symp., Tiergarten Schönbrunn, Vienna, Austria 2001; *in print*.
5. Turner MO, McDonnell SM and Hawkins JF. Use of pharmacologically induced ejaculation to obtain semen from a stallion with a fractured radius. J Am Vet Med Assoc 1995; 206: 1906-8.
6. Walzer C. Dermal anesthesia in the white rhinoceros (*Ceratotherium simum simum*) using a eutectic mixture of lidocaine and prilocaine. J Zoo Wildl Med 1998; 29: 300-2.
7. Walzer C, Pucher H and Schwarzenberger F. A restraint chute for semen collection in white rhinoceros (*Ceratotherium simum simum*) – Preliminary results. European Assoc. of Zoo and Wildl. Vet. (EAZWV), Paris, 2000; 3, Suppl.: 7-10.
8. Walzer C, Göritz F, Silinski S, Hermes R, Hildebrandt T and Schwarzenberger F. Anesthesia management in white rhinos for reproductive evaluation, semen collection and AI – a team approach. Proc. Intern. Elephant and Rhino Res. Symp., Tiergarten Schönbrunn, Vienna, Austria 2001; *in print*.
9. Wielebnowski N. Behavioral differences as predictors of breeding status in captive cheetahs. Zoo Biol 1999; 18: 335-49.

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THE USE OF IMPLANON â CONTRACEPTIVE IN APES

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Abstract

An alternative contraceptive to Norplant was sought to control breeding of chimpanzees at Edinburgh zoo. This paper reviews the successful use of Implanon â, a subdermal etonogestrel implant in chimpanzees *Pan troglodytes* at the zoo.

Zusammenfassung

Eine alternatieve Kontrazeption zu Norplant wurde gesucht, um die Fortpflanzung von Schimpansen im Zoo von Edinburgh zu kontrolieren. Dieser Artikel berichtet über den erfolgreichen Einsatz von Implanon®, ein subdermales etonogestrel Implantat, bei Schimpansen (*Pan trogodytes*) in diesem Zoo.

Résumé

Une alternative à l'usage de Norplant comme contraceptif chez les chimpanzés du zoo d'Edimburgh. Cette présentation présente l'utilisation avec succès d'Implanon, un implant oestrogénique sous-cutané, chez les chimpanzés (*Pan troglodytes*) du zoo.

Key words: apes, contraceptive, implanon, norplant, primates.

Introduction

Different management strategies are applied at zoos for the control of animal populations, inbreeding and hybridisation. Culling may be accepted within the zoo community but not by the general public. Control of population numbers, avoiding inbreeding and hybridisa tion by separating male and female maybe logistically impossible and will to abnormal social structures. Surgical intervention is suitable if permanent sterilisation is wanted and the method used {(2) castration, vasectomy or ovariohysterectomy} will depend on species and group dynamics. Contraceptives are reversible, non-invasive but have a limited duration and can cause undesirable side effects. The AZA contraception database (1,6) has collected data on the following contraceptives in primates: MGA (melengestrol acetate) implants, Depo-Provera (medroxyprogesterone acetate) injection, birth control pills and Norplant (levonorgestrel).
Birth control pills are effective but not reliable as primates will not always accept or swallow the pills. Norplant is a sub-dermal hormone implant containing levonorgestrel distributed in 6 rods. In humans it lasts for up to 5 years in non-human primates the duration is less. The chimpanzee may still have sexual swellings and mate, making it difficult to tell if the implant needs to be replaced. Norplant has been discontinued in the UK since 1999 and a special veterinary import licence is needed. Successful placement and removal of the implant requires some expertise. In view of the above veterinarians have sought new contraceptives. Intra uterine devices (IUD) have been used in some collections. Implanon (4) has been licensed as an intradermal contraceptive since 1999 in the UK and been used at Edinburgh zoo since 2000.

Materials and method

Implanon (3) consists of one rod containing 68mg etonogestrel (3rd generation progestagen). The core consists of ethylene vinylacetate copolymer (46mg) and the skin 15mg ethylene vinylacetate copolymer. In humans the duration of effect is up to 3 years (although it is shorter in heavier women > 75kg). Reported adverse effects and precautions in humans include increased incidence of thromboembolism and hypertension. Headache, acne, increased body weight, alopecia, mood changes and dysmenorrhoea are reported with variable frequencies.
The chimpanzees were anaestethised with a medetomidine and ketamine combination administered by dart. The implant was placed according to manufacturer instruction. The implant is inserted at the inside of the upper arm 6-8cm above the elbow crease in the groove between the biceps and the triceps. The insertion site was clipped and disinfected. 2ml of lidocaine (1%) is injected under the skin along the insertion site. The skin is stretched with thumb and index finger over the insertion site. The needle is introduced under the skin and parallel to the skin surface while lifting the skin with the tip of the needle. The needle is inserted to its full length. Once the implant is placed and the applicator removed a drop of tissue glue is placed on the insertion site. A blood sample was taken for a general health profile from the cephalic vein and the animal was reversed with atipamezole by intramuscular injection.

Results

The following three chimpanzees were implanted with Implanon:
1. Lindsey (date of birth 24 December 1983). Offspring born 22.12.90, 20.2.93 and 5.2.97. Reported cycling again in June 2000. Oral contraception was started in July with Loestrin 20® (ethinyloestradiol 20micrograms and norethisterone acetate 1mg). On the 22nd of October she received her final pill and on the 25th of October 2000 the Implanon was implanted under general anaesthesia (2.25mg medetomidine combined with 225mg ketamine and reversed with 11.25mg atipamezole). No sexual swellings have been observed since.
2. Cindy (date of birth 1964). Offspring born 8.3.93 and 28.3.95. Menstrual cycles reported since April 2000, that same month she started oral contraceptive (Loestrin 20). On the 28th of June 2000 the Implanon implant was placed (following 7 days of oral contraceptive). 2.5mg medetomidine and 250mg ketamine were injected by dart for general anaesthesia and reversed with 12.5mg atipamezole. No sexual swellings have been observed since.
3. Kilimi (date of birth 20 February 1993). First sexual swelling was noted on the 23rd of February 2001. On the 14th of March an Implanon® implant was placed in her arm. General anaesthesia was obtained by dart containing 2.25mg medetomidine and 225mg ketamine and reversed with 2.25ml atipamezole. Estimated weight at 35kg. No sexual swellings have been observed since.
According to manufactures instructions the implant should be inserted preferably on the day following the last active tablet or at the latest on the day following the tablet free interval.
Animals should be considered fertile for at least 2 weeks after placement of an implant in case follicles were present on initiation of treatment (2).

Discussion

Implanon has certain advantages over Norplant for use in the United Kingdom. No import licence is needed and cost is about a fifth of the price. It is faster and easier to implant and presumably easier to remove (5). In women a disturbed bleeding pattern was the most common adverse effect and a reason for discontinuing the two types of implants (7). The incidences were higher in Norplant than Implanon. Normal bleeding pattern resumed 3 months after removal of the implant. No swellings or mating have been observed with Implanon making it easier to ascertain when the implant needs to be replaced. On the down side the duration of efficacy will probably be shorter than Norplant. No information is available if part of a rod can be used in smaller primates such as callithrichids.

References

1. Asa CS and Porton I. Primate contraception methods in use and in development. Proceedings of the American Association of Zoo Veterinarians 1990; 263-4.
2. Asa CS. Contraception. In: Zoo and Wild Animal Medicine, Current Therapy 4. WB Saunders Comp. Philadelphia 1999; 316-320.
3. CBG. Openbaar beoordelings rapport IMPLANON, College ter beoordeling van geneesmiddelen 1999
4. Croxatto HB and Makarainen L. The pharmodynamics and efficacy of Implanon ® - An overview of the data. Contraception 1998; 58: 91-7.
5. Mascarenhas L. Insertion and removal of Implanon ®. Contraception 1998; 58: 79-83.
6. Porton IJ. Results for primates from the AZA contraception database: species, methods, efficacy and reversal. Proceedings of the American Association of Zoo Veterinarians 1995; 381-93.
7. Zheng SR, Zheng HM, Qian SZ, Sang GW and Kaper RF. A randomised multi-centre study comparing the efficacy and bleeding pattern of a single rod (Implanon ®) and a six capsule (Norplant ®) hormonal contraceptive implant. Contraception 1999; 60: 1-8.

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ULTRASONOGRAPHIC DIAGNOSIS OF PREGNANCY IN SOME SPECIES OF REPTILES.

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Abstract

The article describes the ultrasonographic determination of pregnancy in different species of reptiles with a 240 Parus Vet scanner (Pie medical, Maastricht, The Netherlands). The conclusion is that ultrasonography is a very useful tool in the diagnostic evaluation of pregnancy in the different species of reptiles.

Zusammenfassung

Dieser Artikel beschreibt die Trächtigkeitsbestimmung bei verschiedene Reptilienarten mittels Ultraschall mit einem 240 Parus Vet Scanner (Pie medical, Maastricht, The Netherlands). Das Ergebnis ist dass Ultraschall eine praktische Methode für die Trächtigkeitsdiagnostik bei Reptilien darstellt.

Résumé

Cet article décrit la détermination échographique d'une gestation chez différentes espèces de reptiles avec un échographe 240 Parus Vet (Pie médical Maastricht, Pays bas). La conclusion est que l'échographie est un outil très utile pour les diagnostics de gestation chez les différentes espèces de reptiles.

Key words: ultrasound, ultrasonography, reptiles, pregnancy, diagnostics, 240 parus vet scanner, Pie medical.

Introduction

Foetal development in viviparous species as well as postovulatory egg development in oviparous species is described as “pregnancy”.
In snakes and lizards very often pregnancy can be determined by observation of the appearance and behaviour of the individual female (1). By gentle palpation, eggs or developing foetuses can be felt in some species of snakes or lizards. In some lizards eggs can be seen through the semi transparent skin. In tortoises and turtles the hard shell compromises the observations. Sometimes eggs can be palpated in the inguinal fossa.
The reliable method of pregnancy determination can be obtained with radiography or ultrasonography.
In lizards and snakes ultrasonography is the method of choice. In tortoises the limiting factor for ultrasound imaging is the size of the transducer and the size of inguinal openings, just cranial to the hind limbs (the femoral fossa). When this part of the tortoise is large enough, ultrasonography is a good choice (2).
The purpose of this study was to investigate the possibilities of determining pregnancy in various reptile species with a 240 Parus Vet scanner (Pie medical, Maastricht, The Netherlands).

Materials and methods

The species of snake scanned ultrasonographically were three *Corallus enydris hortulana* (grey and black garden tree boa), one *Epicrates cenchria cenchria* (Brazilian rainbow boa), one *Python molurus pimbura* (Ceylonese python), and, two Thamnophis sirtalis (garter snakes). The lizards were three *Tiliqua scincoides* (blue tongued skink), one *Pogona vitticeps* (bearded dragon), two *Physignathus cocincinus*, (Chinese water dragon). The tortoises were two Geochelone carbonaria (red foot tortoise).
All healthy mature female animals were scanned for pregnancy.
Ultrasonic examination was performed using a 240 Parus Vet scanner with a curved array dual frequency probe (7.5 MHz) (Pie medical, Maastricht, The Netherlands).
An ultrasound coupling gel (Pie medical, Maastricht, The Netherlands) was applied to the skin to avoid interposition of air. All individuals were manually restrained and no sedation was used. In snakes the ventral, caudal one third of the body length measured from the mouth to the cloaca, was scanned. In lizards the caudal halve of the body was scanned from the ventral side. The skinks were also scanned from the lateral side. In the tortoises the right and left inguinal openings (femoral fossa), just cranial to the hind limbs were used.

Results

Three out of 3 *C. e. hortulana* were pregnant, although they seemed to be in different stages of gestation (fig. 1 and 2).

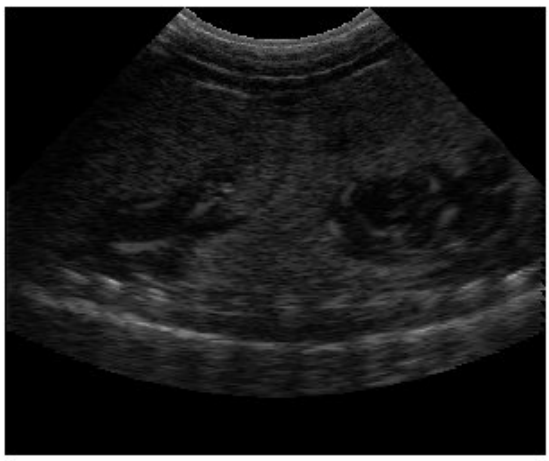

Fig. 1. *C. e. hortulana.* Pregnant, two fetuses can be seen.

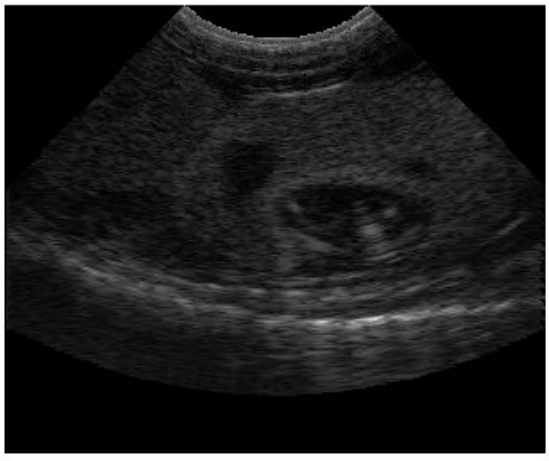

Fig. 2. *C. e. hortulana*. Pregnant, a large yolk portion with a fetus can be seen.

The *E. cenchria cenchria* was also pregnant, which was totally unexpected (Fig. 3). The animal delivered 13 young snakes 39 days after the ultrasound was done.
In only one blue tongue skinks, the heart of a foetus could be seen beating. The female delivered 8 dead, not fully developed and one living young a couple of days after the scan. The other lizards were not pregnant.
One of the 2 *G. carbonaria* proved to be pregnant (fig.4). She delivered the eggs after one injection with oxytocine-S^{R} (Intervet Nederland B.V., Boxmeer, The Netherlands).

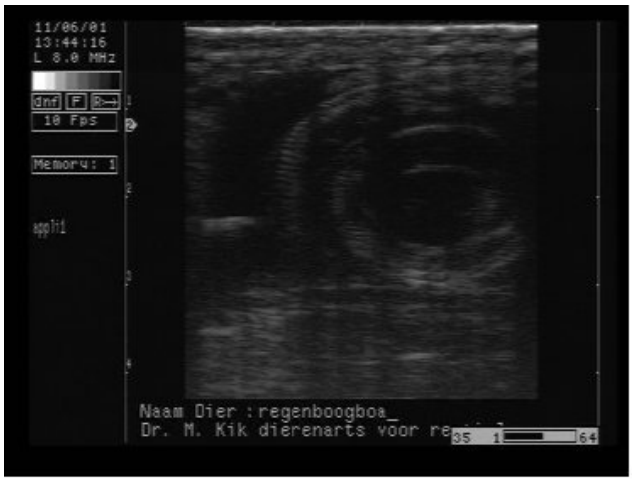

Fig 3. E. *c. cenchria*. A curled up fetus can be perfectly seen.

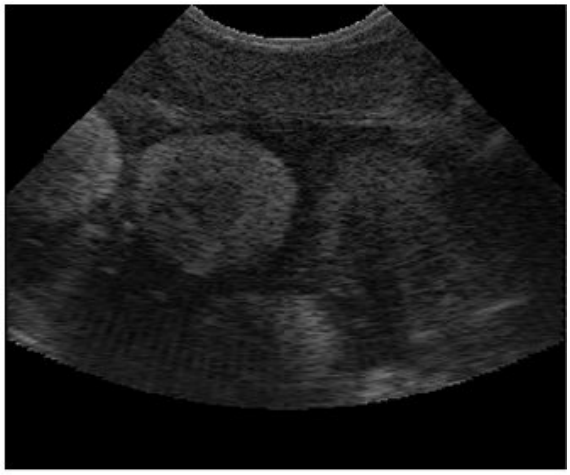

Fig. 4. Eggs in the oviduct of a *G. carbonaria*. Four eggs can be identified.

Conclusion

Ultrasonography is a simple, fast, non-invasive method for the determination of pregnancy in different species of reptiles.
In snakes when the ultrasound coupling gel is copiously administered no interposition of air hindered the imaging. Whereas, in the skinks the gel needed to be applied for a longer period of time prior to scanning. Obviously the scales of the skinks are thicker and more rigid than in snakes.
The size of the femoral fossa of the *G. carbonaria* was big enough for the size of this probe used.
In oviparous snakes, staging of the duration of the pregnancy must be possible if enough animals are followed over time.
In lizards differentiation of pre-ovulatory follicles and eggs in the oviduct is possible.
One disadvantage of ultrasonography in tortoises is, when the number of eggs has to be counted in cases of dystocia. Superposition of eggs is possible and thus the number of eggs can vary.
Ultrasound is a useful tool in the diagnostic evaluation of pregnancy in the different species of reptiles. The 240 Parus Vet scanner (Pie medical, Maastricht, The Netherlands) with a curved array dual frequency probe (7.5 MHz) is very useful for this purpose.

References

1. DeNardo, D. Reproductive biology. In: Reptile medicine and surgery. Ed. D.R. Mader. 1996. W.B Saunders Company, U.S.A. pp. 212-213.
2. Penninck, D.G., J.S. Stewart, J.P. Murphy, and P. Pion. Ultrasonography of the California desert tortoise (Xerobates agassizi): anatomy and application. Veterinary radiology 199132,3: 112-116.

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